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Content

Respected readers,

Journal of Natural Sciences and Engineering (JONSAE) is a peer-reviewed biannual journal that aims at the publication and dissemination of original research articles on the latest developments in the fundamental theory, practice and application of engineering, science, and technology. We provide a platform for researchers, academicians, professionals, practitioners, and students to impart and share knowledge in the form of high-quality empirical and theoretical research papers. The journal covers all areas of genetics and bioengineering, electrical and electronics engineering, information technology, architecture, applied mathematics, computer sciences, and civil engineering.

In this first issue in 2022, I would like to express my gratitude to our authors and manuscript reviewers for their unselfish effort. You have continued your commitment and diligence to the Journal in helping us to produce quality and meaningful content that has the opportunity to advance the field. All of us had personal and professional challenges due to the pandemic and return to in-class teaching and yet despite this, we managed to assure continuous publication of our Journal. Thank you all for being part of this wonderful academic endeavor.

This issue is dedicated to natural sciences only, whereby we have two original research articles and two review articles. Original articles are dealing with testing results for the patients in the Canton Sarajevo, one related to biochemical blood analyses and another to cancer genetics. When it comes to literature reviews, we are changing the pace and are pointing our interest towards plants; the reviews are dealing with plants in medicine and environmental science.

In the end, we plan to build upon the excellent editorial infrastructure in the next years. In addition to managing the normal turnover of the Editorial Board members, we will seek to expand it by recruiting additional members who can provide expertise in areas that are not currently well represented. In particular, we hope to recruit board members with expertise in such areas as statistical and biostatistical methods, computer science, bioengineering, and biomedical engineering, among others. We also hope to leverage the expertise of the Editorial Board members to train the next generation of scholars and potential Editorial Board members by finding ways to pair student reviewers with senior reviewers for a peer-review mentorship as a part of building a better research environment for new scientists. A similar approach is planned for the administrative members of the Editorial Board, with the aim of improving the quality of the overall Journal design and acquiring a modern visual identity.

Having in mind all stated, I wholeheartedly invite you to read this issue and join our team.

Yours sincerely,

Adna Ašić, PhD
Editor in Chief

Phytoremediation: A Green Approach to Fight Heavy Metal Contamination in the Soil

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Literature review

Abstract: *The purpose of this review paper is to present the extent and importance of the problem of heavy metal contamination in the environment, most notably in the soil. Phytoremediation is one approach for tackling this issue through the use of leafy plants for the uptake of excess heavy metals from the soil. An example of such plant species are the members of the Brassica genus which, when put in contact with different heavy metals present in soil, will act to remove them and therefore decrease their concentration. Heavy metals that are mostly observed in this context were copper, lead, and zinc, because of their abundance in different ecological environments. Different plants in general have a satisfactory phytoremediation potential, depending on the plant part, heavy metals in question, as well as used remediation technique. Therefore, introducing Brassica spp. into use would be a good way of enhancing the quality of the environment.*

Keywords: Brassica spp., Bioremediation, Heavy metals, Phytoremediation, Pollution.

1. Introduction

Heavy metal pollution is a growing concern throughout the recent period. Possible solutions were suggested through different scientific methods that require the natural science approach to solve these issues. Bioremediation, a technique used to face the problems regarding environmental contamination, is one of the solutions that scientists aim to implement. This is a method that recycles the harmful waste materials while avoiding any damage to the nature. Additionally, the recycled harmful materials can become a new source of food for different microorganisms. Oil spills are an example of the hazardous material that causes major issues in the ecosystem. Bioremediation is using different microorganisms to resolve these issues, as they are capable to survive different environmental conditions due to their metabolic characteristics [1-3].

The twentieth century was the period in which heavy metals became a major issue in the world because of wars, industrialization improvement, and intensive usage of large-scale heavy metals in different industries. Bioremediation faces this challenge to sustain suitable life conditions for humans [3,4].

Water and soil are the main parts of the Earth, as well as the main factors upon which the quality of the food depends, meaning they should be especially well-protected. Phytoremediation is a technique in which plants are used to eliminate the hazardous materials that are a threat to the soil, water, and air. A variety of physical and chemical methods are used so that metalloids and heavy metals could be removed, whereby a variety of potentially dangerous outcomes could be minimalized [5,6]. This branch of research uses several methods in which the soil quality could be improved, including chemical leaching, soil replacement, and electrokinetic remediation, that are all capable of soil remediation. Phytoremediation is therefore an appropriate green technique that works on eliminating all contaminants and its success depends on the type and concentration of the toxin that is remediated by the plants [7,8].

Different plant species can act to remove the toxic concentrations of zinc (Zn) and copper (Cu), with the most prominent example being the plants from the *Brassica* genus. Certain plant- growth-assisting (PGP) bacteria are capable of enhancing the growth of plants when dealing with different heavy metals. Other plants that were also used in bioremediation studies are *Micromeria cristata, Mentha spicata* var. *crispa, Mirabilis rotundifolia,* and *Celosia argentea.* When studying the phytoremediation capacity of plant species, different factors are analyzed, including the remediation capacity for different parts of the plant, its height, tolerance index, and root and shoot biomass [9,10]. An additional factor that must be accounted for is not to overwhelm the plant

with the potentially toxic metals (PTMs). Long-term effect of toxic metals is observed at increased temperatures, through measurement of biochar production during pyrolysis of biomass in the absence of oxygen. This represents an oxidation resistance whereby small amounts of carbon (C) are lost. As a safe strategy for organic waste management, thermal conversion of phytoremediation plant waste material into biochar can be used. At 350, 550, and 750 degrees Celsius, a potential phytoremediation plant, *Silphium perfoliatum*, was pyrolyzed for biochar synthesis. The oxidation resistance and long-term leaching risk of PTMs in the produced biochar were examined. With the increased pyrolysis temperature, PTMs in biochar might change into more stable and less hazardous forms, according to the findings [11].

Brassica plants are popular species in plant technology research because they are used for different purposes, such as phytoremediation, agriculture, and horticulture. These plant species are used to reduce symptoms that are caused by specific diseases because plants are able to take up these toxins and reduce the harmfulness towards humans. They reduce the concentrations of insect pests that could potentially cause damage to some plants [11,12]. In addition, *Brassica* spp. are used as excellent phytoremediation tool. Heavy metals can appear in the environment as a natural process, for example due to volcanic eruptions or weathering of rocks but can also be man-made. *Brassica* spp. are used in resolving these issues because these plants can adapt to different pH and heavy metal concentrations in the soil, giving them an advantage in further growth and heavy metal uptake [13].

2. Methodology

For this review paper, 25 original research papers were analyzed. All articles were obtained for the purpose of determining the effects *Brassica* spp. when in contact with different heavy metals. The databases from which research data were obtained are Elsevier, PubMed, Springer, and PubMed Central (PMC). In order to be included in this review, the article had to present original research or literature review on phytoremediation capacity of *Brassica* spp., the used species and heavy metal(s) must be clearly identified, used heavy metal concentrations had to be specified, and the experiment outcomes must be clearly presented. This review is a summary of obtained data with the goal of identifying experimental setup in which *Brassica* spp. can act as potent phytoremediator. The time period in which original studies were conducted is from 2014 until 2021, with the conclusion that the usage of *Brassica* spp. for this purpose has increased recently compared to previous period.

2. Phytoremediation and its Applications

A specific critical concentration of each heavy metal is known at which it becomes a serious threat for the environment (Table 1). Lead (Pb) is a toxic heavy metal that can cause extensive health problems to humans. It can be recognized by its silvery texture and has the capability to tarnish with air. A previous study that was done has concluded that approximately 53,500 children between ages of 1-5 will have elevated blood lead concentrations because of the poisoning in their early childhood. Children under the age of one year had consistently lower rates of increased blood lead concentrations than children aged one to four years, owing to the fact that lead is a cumulative toxin and that young children are more mobile and engage in more hand-to-mouth activities than babies. In general, the percentage of individuals with increased blood lead levels is not as severe [14,15]. Lead poisoning has a major impact on human health, since it can cause anemia, among other problems. Lead poisoning experiments in rats showed that if lead nitrate concentration was too high, the rats were suffering from diabetes [16].

Table 1. A comparison of soil concentration and critical concentration of the most commonly encountered heavy metals (taken and adapted from [25]).

Heavy Metal	Source	Concentration in soil (µg/g)	Critical concentration in soil (µg/g)	References
Copper (Cu)	Industrial waste, landfills, agriculture, and phosphate fertilizers	50-100	100	[14,25]
Lead (Pb)	Industrial activities, fertilizers, burning fossil fuels, pesticide manufacturing, and fertilizers	150-200	100	[15,25]
Zinc (Zn)	Mining, mine tailing, discharges of wastes, coal, and fly ash	100-200	100-150	[21,25]

Copper (Cu) is one of the most common heavy metals that are found in waste waters, but it does not disturb certain physiological systems as lead does (Table 1). Copper is needed for proper functioning of human organs and tissues, and can be found in higher concentrations in kidneys, brain, and liver. The essential copper intake in humans is one to 100 mg/day; however, an increased copper concentration in the body is toxic. Copper poisoning can be induced by eating acidic meals cooked in uncoated Cu cookware, or by drinking water or other environmental sources containing too much Cu [17]. Heavy metals not only select for metal resistance, but they can also co-select for

antibiotic resistance, which is a major public health problem. The tests have been performed to measure the heavy metal effect on bacterial resistance in water, where Cu and zinc (Zn) were among the heavy metals found to contribute to this phenomenon, due to their excessive presence in water samples [18].

Zinc is therefore another source of contamination found in mining areas (Table 1). The mining areas were analyzed in China, and they showed that the heavy metal concentrations were above average, meaning that the ecological situation could be threatened if not treated properly. Zn concentration was ranging from 60.44 mg/kg to 4,946.59 mg/kg, and an average of 736.55 mg/kg, which is much above its critical concentration value. Other two heavy metals were also analyzed in the study. Pb concentration was ranging from 54.60 mg/kg to 10,053.90 mg/kg and an average of 777.24 mg/kg, while Cu had concentrations between 6.06 mg/kg and 120.52 mg/kg and an average of 24.18 mg/kg, therefore being the only heavy metal within the accepted limits of concentration in the environmental samples. Such high heavy metal concentrations are also potential sites of phytoremediation with adequate plant organisms. The soil samples in this investigation were neutral to slightly acidic, according to some of the findings. Cu and nickel (Ni) concentrations in the soil were found to be within acceptable limits, while Pb, cadmium (Cd), and Zn concentrations were greatly above the Chinese Soil Environmental Quality Standard's equivalent limitations [21, 22].

In one previous research, *Brassica* was used in the form of a grown plant samples from different areas of Zhejiang province, China, together with the soil sample in which it was growing. Plant samples were washed with tap water and then in distilled water and later tested for the uptake of heavy metals of different concentrations. The salts of heavy metals were detected from the soil in which *Brassica* was grown. The detection limits for Cd, Pb, arsenic (As), mercury (Hg), and Cr were 0.001, 0.005, 0.002, and 0.01 mg/kg, respectively. The Chinese cabbage (*Brassica campestris* spp. Pekinensis), pakchoi (*Brassica chinensis* L.), celery (*Apium graveolens*), cherry tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*), cowpea (*Vigna unguiculata*), pumpkin (*Cucurbita pepo* L.), and eggplant (*Solanum melongena*) were used in the mentioned experiment. The average values of Cd, Pb, and Cr in 97 vegetable samples, including those from the *Brassica* family, were 0.020, 0.048, and 0.043 mg/kg, respectively [22].

In another study, plants were also tested in order to check for their heavy metal absorption abilities. The plants have been tested on different types of soil that contained

a variety of heavy metals, namely Hg, Pb, Cd, and Cr. The cabbage plant from the *Brassica* genus that has been tested was from China, where the heavy metals have a major impact on the soil and water contamination. The procedure was to carefully wash and prepare the entire vegetables with deionized and tap water so that all excess materials could be removed. The samples were digested in a protocol using nitric acid and hydrogen peroxide, followed by the transfer of the digests into a volumetric flask and filtration. In order to determine heavy metal concentration in the plants, an inductively coupled plasma-mass spectrometry was used. The bio- concentration factors (BCF) have been used to compare the pollutant concentration in the soil environment with the plants that contained the pollutant residues. The results of this study imply satisfactory phytoremediation capability of *Brassica* spp., while the best results were more generally obtained with leafy vegetables. Metal concentrations in vegetables and the soils in which they grew demonstrated a strong positive association, especially for green plants like cabbage. The BCF for Cd is higher than that of Pb and Cr. As a result, heavy metal pollution in leafy and stem vegetables, notably Cd, should be given more attention [22].

The process of phytoremediation encompasses several different processes, some of them being particularly useful in clearing the metal waste from the environment, while others are aimed at dealing with other types of waste such as organic pollutants. For example, the process of rhizofiltration is aimed almost exclusively at plant roots adsorbing and absorbing metals as pollutants, whereas phytodegradation is a process of degradation of organic pollutants, such as DDT [5]. Different species of plants were used for processes of phytoremediation involving removal of metal pollutants, and a summary of their main characteristics can be found in Table 2, in particular with respect to *Brassica* species, which is in focus of this review.

Table 2. Advantages and disadvantages of different plant species used for soil bioremediation with focus on metal decontamination.

Process in phytoremediation of metal pollutants	**Process explanation**	**Typical pollutants**	**Measurement**	**Plant used and its characteristics (advantages or disadvantages)**
Phytoextraction	Uptake of contaminants by plant roots and their accumulation in shoots	Cd, Pb, Zn	Shoot metal concentration and shoot biomass [26]	*Brassica juncea* - accumulates metals to a lesser degree but produces more biomass above ground. This is a disadvantage because it makes the plant less safe to handle when disposing large amounts of the accumulated metals, as well as costly due to large biomass *Trifolium spp.* – offer multiple harvests throughout the growth time, high adaptability to stress conditions [27]
Rhizofiltration	Roots absorb and adsorb pollutants (mainly metals)	Zn, Pb, Cd,	Not quantified directly	*Zea mays* - a high potential for absorption and accumulation of mercury [29] *Brassica campestris* – high potential for accumulation of uranium [30]
Phytostabilization	Reducing the bioavailability of pollutants in the environment (e.g., through changing its oxidative state)	Cu, Cd, Cr, Ni, Pb, Zn	Oxidative state of the metal in question	*Brassica juncea* – good tolerance for PTE toxicity *Dactylis glomerata* – functions better in a less contaminated soil. Both plants work better through their roots, rather than shoots [31]

4. Conclusion

Brassica spp. is offering very promising results regarding its ability to remove heavy metal contaminants from the environment. It was shown effective for absorbing different metal concentrations and different elements. A variety of plants are promising for the purpose of reducing heavy metals in the environment by means of phytoremediation. Since this technique is generally getting more interesting for the research community, more experimental results for *Brassica* spp. and other plant species are necessary to assess their optimum usage conditions as phytoremediators. In addition, certain areas of the world are representing the heavy metal concentrations significantly exceeding the critical values. Plants are an excellent tool to reduce the heavy metal concentrations and create a healthier ecological environment for microorganisms and animals. Further research is needed to improve the usage of phytoremediation technique for tackling this issue.

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The Presence of Bioactive Compounds in Plants of the Amaranthaceae Family and Their Use in Medicine: A Review

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Literature review

Abstract: *Various plants have been used in traditional medicine for thousands of years as natural medicines with therapeutic and other pharmacologic effects. Bioactive compounds found in plants, such as flavonoids, trace minerals, essential oils, phenols, glycosides, alkaloids, and tannins, can affect microbial growth, reproduction, and essential cell functions. Plants of the Amaranthaceae family have a broad range of bioactive phytochemical constituents, which provide a variety of medicinal benefits. This review article discusses the characteristics of Amaranthaceae plants that may indicate their use as medicinal plants, especially against infectious diseases. According to the literature, Amaranthaceae plants contain considerable levels of bioactive compounds that make them effective in traditional medicine, even* though their impact on numerous microbes has yet to be examined.

Keywords: Active compounds, Amaranthaceae, antioxidant, antibacterial, flavonoids.

1. Introduction

Since ancient times, different plants are utilized in traditional medicine as natural medicines with therapeutic and other pharmacologic effects. According to the World Health Organization (WHO), traditional medicine is used by up to 80% of the world population for main healthcare needs. The first findings of a WHO study reveal that the number of people who use medicinal herbs is considerable and growing, even among the young population. In addition, the World Health Organization recommends medicinal plants as the best source of a wide range of drugs [1, 2]. Plants or some of their parts, such as leaves, roots, seeds, and flowers, can be used in a variety of ways, including fresh crude form and teas, decoctions, powder form, or extracted forms [2].

2. Bioactive Compounds

Plants contain active components that may impact the growth of microorganisms, reproduction, or some essential cell activities. Some examples of active compounds include phenols, flavonoids, trace minerals, essential oils, glycosides, alkaloids, and tannins, which could be used in the production of drugs. The extracts of medicinal plants are also utilized as preservatives in food, preventing harmful microorganisms from growing [2, 3]. Flavonoids and phenolic compounds are characterized as an effective antioxidants, anticancer, antimicrobial agents, anti-inflammation, immunological response boosting agents, skin protection agents, and potential pharmaceutical and medical agents. Due to numerous advantages for human health, research dealing with flavonoids and other phenolic compounds from medicinal plant species has increased significantly over time [4]. Plants and their bioactive components play a significant role in the search for new antimicrobial agents. Specific phytomolecules have been shown to block bacteria's quorum-sensing function, suggesting that they could be utilized to treat infections associated with bacterial biofilms [5]. Aside from medical uses, medicinal plants may be beneficial to human nutrition because they also contain various bioactive chemicals such as vitamins and essential oil components. Because of the presence of antioxidants and antibacterial elements, as well as the flavoring and coloring qualities of several medicinal plants, they are usually utilized in food products and cosmetics [2].

3. Amaranthaceae Family

There are more than 165 genera and 2040 flowering plant species in the Amaranthaceae family, which can be found all over the world, from tropical to cold temperate areas [6]. Within the flowering plant order Caryophyllales, it is the most species-rich lineage. The Amaranthaceae family includes annual and perennial

species, predominantly herbs, as well as bushes, tiny trees, and grapevines. Several species were used as medicinal plants by natives in tropical and subtropical regions, as well as in temperate climates, for a variety of purposes [7]. Research has revealed that Amaranthaceae extracts have antioxidant [8, 9], antidiabetic [10, 11], immunostimulatory, antitumor, antimicrobial, analgesic [12], anti-inflammatory [13], hypolipidemic [14], diuretic [15], antihypertensive, and hypoglycemia [16] properties. Also, they contain biologically active substances such as betacyanins, flavonoid compounds, phenols, volatile oils, diterpenes, etc. [7, 17, 18]. The most prominent genera and species from the Amaranthaceae family are amaranth (*Amaranthus*), smotherweed (*Bassia)*, chard (*Beta vulgaris*), cockscomb (*Celosia)*, glasswort (*Salicornia*), globe amaranth (*Gomphrena globosa*), goosefoot (*Chenopodium*), orache (*Atriplex*) and spinach (*Spinacia oleracea*) [18, 19].

Amaranth (Amaranthus)

Amaranthus, often known as Green Amaranth, is described in Greek as "never fading." Most plants in the genus are usually thought of as weeds, however, others are grown as leafy vegetables in various regions of the Earth, while phytonutrients that participate in the reduction of free radicals have been observed in various parts of plants [20]. Phytochemical investigations of these vegetables have shown the presence of phytochemicals involved in radical scavenging activities, such as flavonoid compounds, alkaloid compounds, tannins, phenols, glucosides, and glycosides [21, 22]. *Amaranthus* is characterized as an antioxidant, antibacterial, anti-inflammatory, antimalarial, antidiabetic, anticancer, and hepatoprotective agent [23, 24].

Stintzing et al. (2004) used quantitative and qualitative analyses of phenolic compounds and betalains from stem extracts to confirm the antioxidant properties of *Amaranthus spinosus* L. The major betacyanins detected in *A. Spinosus* L. were amaranthine and isoamarantine, and hexacinamates, quercetin, and kaempferol glycosides [25]. Kraujalis et al. (2013) also investigated the antioxidant characteristics of solid plant material and discovered that amaranth leaves and flowers, as well as their extracts, have high antioxidant activity [26]. El-Shabasy & El-Gayar (2019) compared six species of the Amaranthaceae family (*Aerva javanica, Aerva lanata, Amaranthus graecizans* ssp. *silvestris*, *Amaranthus hybridus* L., *Amaranthus viridis* L., and *Digera muricata* L.) based on their antimicrobial properties and concluded that they have an antimicrobial effect against some pathogenic bacteria [27].

Smotherweed (Bassia)

Bassia is an annual plant genus in the Amaranthaceae family containing about ten species native to Eurasia. Many *Bassia* species can survive in saline soil and are toxic to grazing animals, especially sheep. The five-horn smotherweed (*Bassia hyssopifolia*) and hairy smotherweed (*Bassia hirsuta*), both brought to the Americas, are considered invasive in locations outside of their native habitat [28]. The genus' members are generally plants or subshrubs with dense hairs covering them. The slender leaves are sessile (meaning they don't have a leafstalk) and alternate along the stems. The bisexual blooms are produced in terminal spikes with unique hooked or conical appendages. Achenes with little brown seeds are the fruits [28, 29]. The chemical content of *Bassia* extracts was primarily studied for pharmacological investigations, to detect biological activities and develop new pharmaceuticals. According to traditional medicine, plants of this genus exhibit antiparasitic, cardioprotective, tonic and other properties [30]. Scientific and clinical studies examining hypotensive, hypolipidemic, anticarcinogenic, analgesic, and other effects [31, 32, 33], have shown the presence of flavonoids, tannins, saponins, and alkaloids for the species *B. prostrata, B. scoparia*, and *B. muricata* [34–37].

The presence of eleven phenolic compounds in *Bassia prostrata* plants was investigated and confirmed by Petruk et al. (2021), and the quantitative amount of each chemical ranged from 0.1 to 10.8 mg/g [30]. Al-Snafi (2018) stated that the fruit of Kochia scoparia (*Bassia scoparia*) was used in China to treat skin, urinary tract, and eye disorders, as well as in Japan as a meal. It was also commonly used in Southeast Asia to treat painful urination, skin issues, breast pain, and malignancies, as well as a food supplement and treatment for inflammatory conditions like arthritis and chronic pain. Kochia scoparia was applied in traditional Korean remedies as a tonic, diuretic, analgesic, and antidote [34]. Significant amounts of flavonoids and saponins in aerial parts of *B. muricata* were isolated in a study done by Kamel et al. (2001) [35]. Also, Shaker et al. (2013) have demonstrated the presence of flavonoids and saponins in *B. muricata* and proven the antioxidant power of this plant [36].

Chard (Beta vulgaris)

Beta vulgaris L. is native to southern and eastern Europe as well as northern Africa. It can be found in Europe, Asia, America, and Africa. Because it contains a variety of nutrients and biologically active compounds, as well as high levels of antioxidants, vitamins, and other components with health-promoting effects, *B. vulgaris* is very often used in diet [38]. The leaves of chard (*Beta vulgaris* L. subsp. *vulgaris*) contains high levels of vitamins A, B, and C, as well as calcium, iron, and

phosphorus. *B. vulgaris* species are also used in traditional medicine for liver and kidney problems, immunological and hematopoietic system activation, and as a special diet in cancer treatment. They also lower blood pressure and improve endothelial function [38, 39]. Chard leaves are rich in phytochemicals and antioxidants such as flavonoids, phenolic acids, pigments, and certain volatile compounds like anethole, which has an antibacterial and antifungal impact [40].
Pyo et al. (2004) were the first to show that the methanol extract of chard contains both phenolic acids and flavonoids as antioxidant components. They concluded that the antioxidant activity of each chard extract may be connected to their phenolic concentration [41]. Sacan and Yanardag (2010) investigated the antioxidant capabilities, acetylcholinesterase inhibitory capacity, and proline content of chard. Their findings were compared to natural and manufactured antioxidants, showing that chard could be a natural source of antioxidants, antiacetylcholinesterase, and proline [39]. Mzoughi et al. (2019) looked at the chemical properties of chard leaves as well. The findings of this study emphasized the wild Swiss Chard's potential medical advantages as a source of dietary and biologically active components [40].

Cockscomb (Celosia)

The name *Celosia* comes from the Greek word *kelos*, which means "burned," and describes flower heads that resemble flames. If the blooms are crowned with fasciation, they are known as wool-flowers, brain celosia, or cockscombs. The *Celosia* species are used in traditional medicine to treat a variety of conditions, including fever, diarrhea, mouth ulcers, irritation, injuries, and infections [42, 43]. Triterpenoids, saponins, betalains, alkaloids, phenolic compounds, tannins, flavonoids, glycosides, sterols, etc., are among the phytochemical compounds isolated from *Celosia* species [44–48] and they contribute to antiinflammatory, immunostimulatory, anticarcinogenic, hepatoprotective, antioxidant, tissue repair, hypoglycemic, antinociceptive and antimicrobial activity [47–50].

Celosia is a genus of roughly 60 species endemic to subtropical and temperate regions of Africa, South America, and Southeast Asia. *C. argentea* seeds are used in Chinese herbal medicine to treat ocular and liver problems. Also, dried mature seeds are used to cure diseases including hepatitis, hypertension, and sarcoptidosis, as well as to enhance vision [51]. In the research conducted by Hakawa et al. (1998), the anticarcinogenic effect of *C. argentea* seed extract was confirmed. It is dependent on immunomodulation features such as cytokine induction, which resulted in a Th1 dominant immune state, stimulating macrophages to a tumoricidal state, preventing cancer spread [52]. It is also reported that
C. argentea contains flavonoids, which have antiproliferative properties against a

variety of human cancer cells [53, 54]. Rub et al. (2016) analyzed the phytochemical composition of *C. argentea* and found flavonoid and phenolic compounds, both of which are effective scavengers of reactive oxygen species, and whose activity might be used to support the anticancer and antioxidant potential of this plant [55].

Glasswort (Salicornia)

In Europe, the genus *Salicornia*, particularly the tetraploid species, is often used as a vegetable. The seeds are high in oil, and various trials have been conducted in the United States to commercialize tetraploid species, particularly *S. bigelovii*, as a source of vegetable oils [56]. Some *Salicornia* species are used in the diet [57], as well as in the treatment of respiratory problems, arthritis [58], stroke, cirrhosis [59], and diarrhea [60], and have biological activities including antioxidant, anti-inflammatory, antidiabetic, and cytotoxicity [61, 62, 63]. Some of the bioactive compounds found in plants of this genus are fatty acids, sterols, saponins, flavonoids, and phenols [64–68].

S. ramosissima is an annual plant widely distributed in Portugal, France, and Serbia, and according to Isca et al. (2014), it contains a variety of phytochemicals (sterols, mono and polyunsaturated fatty acids, dicarboxylic acids, and alcohols) that are within the safe limits set by numerous international organizations [69]. Proteins, fibers, minerals, and polyunsaturated fatty acids are all found in *S. ramosissima* [70], so it is often used for the production of food supplements and in nutrition [69]. Phenolic substances including quercetin3-O-glucoside and caffeoylquinic acids have also been discovered in this plant, and they are known to help prevent diseases like cancer, hypertension, and cardiovascular disease [70]. Furthermore, the study of Ferreira et al. (2018) supports plant usage for medicinal purposes. They conducted toxicological tests on *S. ramosissima* on mice testis and concluded that it had the therapeutic potential for the male reproductive system, owing to the antioxidant activity of its ingredients [71]. Surget et al. (2014) found that *S. ramosissima* is rich in phenols and flavonoids, indicating that it has antioxidant properties as well as a UV photoprotective impact [72].

Globe Amaranth (Gomphrena globosa)

Globe amaranth, or *Gomphrena globosa*, is an annual branching plant that is grown as an ornamental flowering herb in the garden. It grows well in Bangladesh and is endemic to America and Asia [73]. Its leaves and blossoms have been used in folk medicine to treat high blood pressure, diabetes, renal and respiratory issues, and diseases of the reproductive organs [74, 75]. Biological activities were observed for extracts of various *Gomphrena* species such as antioxidant, antifungal, larvicidal,

antibacterial, anticancer, and estrogenic activities [73, 76–78]. Phytochemical investigations revealed the presence of saponins, alkaloids, betacyanins, hydroxycinamamides, and flavonoids, including flavones and flavonols [78, 79, 80].

Pomilio et al. (1992) analyzed extracts and components of *Gomphrena martiana* and *Gomphrena boliviana* against Gram-positive bacteria (*Staphylococcus aureus, Streptococcus faecalis, Micrococcus luteus* and *Bacillus subtilis*), Gram-negative bacteria (*Salmonella newport, Salmonella oranienburg, Escherichia coli* B, *Escherichia coli* K 12, *Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa and Proteus vulgaris*), spore-forming Gram-positive bacteria (*Clostridium tetanii, Clostridium sporogenes* and *Clostridium butyricum*, an acid-fast bacterium (*Mycobacterium phlei*), a fungus (*Aspergillus niger*), and two yeasts (*Saccharomyces cerevisiae and Candida albicans)*. According to the findings of this research, these plants may potentially be applied in traditional medicine against some infectious agents [78]. Bioactive substances such as saponins, amino acids, nonreducing sugars, flavonoids, etc., were isolated from the methanolic extract of *G. celosioides* by Dosumu et al. (2010). The biological activities of *G. celosioides* extracts were evaluated on a variety of microorganisms; *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli*, and *Salmonella typhi* were all inhibited by the ethyl acetate and methanol extracts of the plant, while the methanolic extract was effective against *Candida albicans, Aspergillus niger*, and *Trichophyton species*. The findings of this study backed up the traditional use of *Gomphrena celosioides* to cure infectious disorders [81].

Goosefoot (Chenopodium)

Although most *Chenopodium* species are colonial annuals, the genus *Chenopodium* contains over 250 species that include herbaceous, suffrutescent, and arborescent perennials. Because of their high protein content and well-balanced amino-acid range, they have been used for ages as leafy vegetables as well as a significant grain crop for human and animal use [82]. Polysaccharides, amines and amides, phenols, flavonoids, saponins, sterols, essential oils, and other biologically active compounds [83–86] isolated from *Chenopodium* species are responsible for a variety of pharmacological properties, including antibacterial, antifungal, anthelmintic, antioxidant, antihepatotoxic, neuroprotective, anti-inflammatory, sedative, and more [87–89].

Sood et al. (2012) studied *C. album* leaves and discovered that they contain phenolic compounds that function as anti-nutrients by binding minerals, but they also have antioxidant properties. The presence of flavonoids and alkaloids in the leaves was

also discovered and they have antioxidant properties as well [82]. *C. ambrosioides*, on the other hand, is used to treat injuries, pulmonary issues, inflammation, bronchitis, tuberculosis, and rheumatic diseases [90]. TrivellatoGrassi et al. (2013) studied the antiinflammatory, nociperception, and wound-healing characteristics of ethanolic extracts of this specimen's leaves and stems, and their findings supported the use of this plant as an anti-inflammatory, pain, and wound-healing treatment [91]. *C. botrys* is also a good example of a medicinal plant from the *Chenopodium* genus. Although *C. botrys* is widely used in folk medicine, most of its medical applications and therapeutic properties are not supported by scientific evidence. Flavonoids, alkaloids, and numerous terpenoids found in the herb [92] have antiparasitic, antifungal, sedative, and analgesic qualities [93]. *C. botrys* bioactive components also demonstrated efficacy against multiple tumor cells, implying that it could be a promising new potential plant for treating cancer [94, 95], though more research is needed to validate this.

Orache (Atriplex)

Atriplex L. species, often known as garden orache, red orache, or simply orache, belongs to the Amaranthaceae family. They are available in Canada, the United States, Australia, and New Zealand, as well as throughout Europe [96]. The genus *Atriplex* L. has over 270 species, many of which are used as antifungal, antidiabetic, and respiratory diseases treatments. This genus includes *Atriplex hortensis*, a halophyte that is used in food products, for treating disease in humans, and for beekeeping [97]. In traditional medicine, aerial parts are often used to treat pulmonary, gastrointestinal, and urinary disorders [98, 99]. The leaves of *Atriplex hortensis* are the most valued portion of the plant, which is regarded to be one of the earliest cultivated plants. Also, this plant is high in vitamins and biologically active compounds [100]. According to literature, bioactive compounds including tannins, phenols, alkaloids, flavonoids, and saponins were found in *Atriplex* species [101, 102].

Bylka et al. (2001) were the first scientists to isolate flavonoid sulfates from leaves of *A. hortensis* and confirmed their presence [103]. According to the literature, flavonoid sulfates have shown many pharmacological properties, such as anticoagulant, anti- inflammatory, and anticancer [104, 105, 106]. Also, Tran et al. (2022) confirmed the presence of flavonoid glycosides in *A. hortensis* var. *rubra* (red orache) [107] which are the essential phytochemicals in our diets. The antioxidant, anticancer and antitumor, hepatoprotective, anti-inflammatory, anti-diabetes, antiviral, antibacterial, and antifungal activity were all demonstrated in flavonoid glycosides [108, 109]. In addition to flavonoids, the presence of phenolic

compounds (e.g. phenolic acids) in *A. hortensis* has been confirmed [96, 98]. Phenolic acids in plants play a variety of roles such as nutrient absorption, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy [110]. They exhibit antibacterial and antiviral properties, as well as significant antioxidant effects due to the action of the hydroxyl group on their aromatic rings, and as a result, they prevent lipids and other molecules from oxidizing [111].

Spinach (Spinacia oleracea)

The plant *Spinacia oleracea* L. is usually referred to as "spinach." It is a 30–60 cm tall herb that is grown as a leafy vegetable all over the world. Some portions of spinach have been utilized in folk medicine to treat a variety of ailments [112]. It contains significant amounts of vitamins, minerals, proteins, omega-3 fatty acids [113] and many bioactive compounds which have been proven to have antioxidant, antiinflammatory, antimutagenic, antitumor, and chemopreventive properties [113, 114, 115].

Adapa et al. (2018) analyzed and confirmed the antimicrobial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* strains of aqueous and ethanolic spinach extracts [112]. In research done by Iqbal et al. (2012), the antibacterial properties of *Spinacia oleracea* extracts were tested against nine mammalian pathogens: *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Pasteurella multocida, Lactobacillus bulgaricus, Micrococcus luteus, Klebsiella pneumonia, Proteous vulgaris,* and *Staphylococcus epidermidis*. Except for *S. typhimurium* and *M. luteus*, all pathogens tested were inhibited by the aqueous spinach extracts. All of the bacterial strains examined were considerably inhibited by the ethanolic extracts, although the n- hexane extracts were particularly efficient against *S. typhimurium* and *S. aureus*. In comparison to other pathogens, sonicated n-hexane extracts exclusively suppressed *S. typhimurium* growth. According to this research, spinach could be a source of novel antibacterial agents [116]. Phenolic and flavonoid contents, as well as antioxidant activity, are confirmed in the study done by Atanassova et al. (2018). Proteins, lipids, insoluble dietary fibers, carbohydrates, and sugar levels were also evaluated, and the results suggest that spinach may offer considerable health benefits and help to prevent chronic diseases [96]. Altemimi et al. (2017) noted that spinach is also rich in defensins that exhibit antimicrobial activities. Defensins in spinach leaves are active against fungi, as well as Gram-positive and Gram-negative bacteria. The goal of this study was to recover antimicrobial compounds from spinach leaves and determine the antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus*. They concluded that spinach leaf extracts have bactericidal properties by triggering DNA

mutations in bacteria and disrupting their cell walls [117].

4. Conclusion

Medicinal plants have been used in folk medicine for thousands of years, and numerous scientific studies have confirmed their effects. The key to their application lies in the presence of various bioactive substances such as phenols, flavonoids, trace minerals, essential oils, glycosides, alkaloids, tannins, etc., the content of which has been proven in the Amaranthaceae family of plants.

This paper presents an overview of 9 plants (*Amaranthus*, *Bassia, Beta vulgaris, Celosia, Salicornia, Gomphrena globosa, Chenopodium, Atriplex*, and *Spinacia oleracea*) from the Amaranthaceae family. Each of them has shown to have a high proportion of bioactive substances, thanks to which they are applicable in the treatment of various diseases or for the preparation of pharmacological products.

Most of these plants, such as *Bassia, Beta vulgaris, Salicornia, Chenopodium, Atriplex*, and *Spinacia oleracea,* are used in the diet because of their high-nutrient ingredients. Also, all of the mentioned plants have antioxidant activity, while only *Amaranthus, Beta vulgaris, Celosia, Chenopodium, Atriplex*, and *Spinacia oleracea* have been shown to have antibacterial activity. Interestingly, anticancer effects of certain species, such as *Amaranthus, Bassia, Beta vulgaris, Celosia, Salicornia,* and *Atriplex*, have also been mentioned in the literature, but more research is needed to prove their effectiveness. Other applications of these herbs in medicine are mainly related to the prevention of skin diseases, respiratory tract diseases, urinary tract diseases, liver diseases, high blood pressure, diarrhea, diabetes, and cardiovascular diseases.

Since Amaranthaceae plants have been successfully tested for various biological activities, and some of their parts have been identified with a high content of biologically active compounds, it can be concluded that these plants are very important in medicine. Most studies have been performed to confirm their antioxidant, anticancer, and anti- inflammatory effects, while only a few studies have tested the antimicrobial activity of these species. Due to the growing number of infectious diseases, it would be good to do tests of different plant species on different types of infectious agents. Also, more research is needed on the direct effect of bioactive substances on diseases of organs such as lungs, stomach, skin, reproductive organs, etc.

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Comparison Test of Sensitivity Between Next Generation Sequencing (NGS) Hotspot Panel and Droplet Digital PCR of KRAS G12 / G13 Mutation

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Orginal research

Abstract: Cancer is an abnormal proliferation of cells that is characterized by the presence of genomic alterations including DNA mutations, deletions, insertions, translocations, inversions, and others. *KRAS* gene is one of the most mutated genes across different cancer types. The most common mutations in *KRAS* are found in codons 12 and 13 of KRAS protein, which are associated with a lack of response to anti- epidermal growth factor receptor (EGFR) antibody therapy. This study assessed and compared the performance between two diagnostic methods: droplet digital PCR (ddPCR) and next generation sequencing (NGS). The main goal was to determine *KRAS* G12 / G13 mutant allele fraction using NGS and to compare the accuracy toddPCR. A total of 28 samples of non – small cell lung cancer (NSCLC) and colorectal cancer (CRC) were analyzed using ddPCR and NGS methods. Our results show that even though both methods exhibited high rate of concordance and correlation, the study proved that ddPCR is more superior when it comes to detecting low frequency mutations. Even though strong correlation was observed, based on the values obtained, we concluded that ddPCR is more accurate, reliable, and sensitive in comparison with NGS.

Keywords: ddPCR, KRAS mutation, NGS.

1. Introduction

It has been determined that *RAS* mutations are the most prevalent oncogenic alteration in human cancers. *KRAS*, followed by *NRAS,* is the member of *RAS* family that is most often mutated. Cancers of urogenital system, colorectal system, lung and pancreas all exhibit the *KRAS* mutation. The enormous heterogeneity that primary tumors with the *KRAS* mutation express has a significant effect on the frequency of the variant allele. This could lead to the metastases' erratic branching progression [1]. *KRAS*gene is recognized as the most frequently mutated oncogene in humanswith the majority of these mutations affecting codons 12 and 13 [2].

KRAS is a significant predictor of anti-epidermal growth factor receptor therapy response, and determining *KRAS* mutational status is critical for successful adenocarcinoma monitoring and treatment. Due to the advances made in field of regenerative medicine, personalized and target therapy it is of vital essence to accurately detect the presence of *KRAS* mutation as precise as possible [3].
The detection of *KRAS* mutation is mostly performed on archived surgical tumor tissue samples or tumor biopsy samples using traditional point mutation detection methods (quantitative PCR and amplification refractory mutation system) or sequencing technique (Sanger sequencing) [4 – 6].
In the past few years however, an increasing number of techniques and detection kits are being developed to detect *KRAS* mutations [7].

The water – in – oil emulsion of the PCR reaction mixture is achieved using droplet digital PCR (ddPCR) technology. This makes it possible to massively sub – partition the reaction into hundreds to millions of separate reactions, creating a synthetic enrichment effect that dramaticallyimproves the ability to detect uncommon mutations present in the sample at very low levels[8].
NGS, on the other hand, offers a low-cost, high-sensitivity, high-throughput platform for investigating genetic abnormalities in the clinical setting [9]. Given the inefficiency of traditional methods in terms of sample, cost, and time, the NGS platform has significant clinical potential for performing such multiplex genetic testing in FFPE CRC specimens [10].

In this study, we wanted to compare the *KRAS*G12/G13 mutation status using tissue and plasma samples of NSCLC and CRC in order to compare the efficiency of ddPCR with that produced by next – generation sequencing.

2. Materials and Methods

Patient samples

A total of 28 samples were included in this study, where 10 samples were from patients with colorectal cancer, 15 samples were from patients with non-small cell lung cancer, and 3 samples were plasma samples from patients with NSCLC. All samples and diagnostic procedures were carried out at Alea Genetic Center, Sarajevo, Bosnia and Herzegovina. The Ethics Committee decision was obtained from International Burch University, Number 04 – 117/21. The study was carried out according to the Declaration of Helsinki.

KRAS mutation detection

DNA isolation from FFPE and plasma samples was carried out using standardized protocols given by the manufacturers. Briefly, deparaffinization of the FFPE samples was done in xylol for 30 minutes, followed by alcohol washing and drying.QIAamp DNA FFPE Tissue Kit was subsequently used for DNA isolation [11]. For the isolation of DNA from plasma samples, QIAamp Circulating Nucleic Acid Kit [11] was used on 1.5 – 2 ml of plasma. After the isolation, the quantification of the isolates was done using QubitFluorometric Quantification under the category of high double – stranded DNA [12]. All samples were stored at -20°C until the further analysis.

Next – Generation Sequencing

All samples were selected based on the presence of *KRAS* mutations in codons 12 and 13, with variant allele frequency ranging 0.6% up to 50.8%. *KRAS* mutational status was determined using Colon/Lung hotspot panel on Ion GeneStudio™S5 instrument. The primary source was analyzed by Torrent Suite Software 5.8.0. The sequences were paired in accordance to the h19 human reference genome. The variants detected were analyzed using ClinVar platform as a referent database. NGS libraries were prepared applying Ion AmpliSeq™Library Kit 2.0 and Ion AmpliSeq™Colon and Lung Cancer Research Panel v2 primers according to the guidelines [13]. This is a hotspot panel intended to identify statistically significant regions of genes, including *KRAS* mutational hotspot regions [13].

Droplet Digital PCR

KRAS tissue genotype analysis was performed using ddPCR *KRAS* G12 / G13 Screening Kit assay which covers codon 12 and 13.

The reaction mixture required for ddPCR contained the ddPCR*KRAS* G12 / G13 screening kit assay which included: Bio – Rad ddPCR™Supermix for Probes and Bio – Rad ddPCR™*KRAS* G12 / G13 Screening Assay. The final volume of 20 µL consisted of extracted DNA, SuperMix for probes (10 µL), *KRAS* screening assay (1 µL) and distilled water which was added in accordance with the amount of DNA sample included in the reaction. The droplet generator partitioned each sample in the well into nearly 20, 000 nanoliter – sized droplets, which were further amplified in a conventional 96 – well PCR plate.

The reaction conditions were set as following: enzyme activation (95°C for 10 minutes and 1 cycle), denaturation (94°C for 30 seconds and 40 cycles), annealing / extension (55°C for 1 minute and also 40 cycles), enzyme deactivation (98°C for 10 minutes and 1 cycle) and hold as an optional step at 4°C. After that, the droplets were transferred to the Bio – Rad QX200™ Droplet Reader.

The QuantaSoft Software was used to analyze the generated droplets using a two – color detection system FAM and HEX / VIC [14]. The software is designed to classify the droplets into three categories: wild type, mutants and empty droplets which, based on the level of fluorescence threshold, exhibit no target.

Statistical Analysis

The two-sample t-test was used to compare mean mutation frequency values between NGS and ddPCR obtained results.

3. Results

Clinical characteristics

The clinical features of patients diagnosed with colorectal cancer and non – small cell lung cancer, the type of *KRAS* mutation, and mutant allele fraction as determined by NGS and ddPCR platforms are shown in Table 1. Briefly, the study included 16 (57 %) male and 12 (43 %) female patients. The patients' average age was 66 (67 for male patients and 66 for female patients).

Table1. Clinical features of patients diagnosed with cancer and *KRAS* sensitivity results.

Sample Number	Age	Gender	Sample Type	Analysis Type	*KRAS* Mutation	NGS Frequency	ddPCR Frequency
1	1949	F	FFPE tissue	NSCLC	GLY12ALA	30%	42%
2	1950	M	FFPE tissue	NSCLC	GLY12ALA	3.2 %	0.72 %
3	1962	M	FFPE tissue	NSCLC	GLY12CYS	13 %	21.3 %
4	1950	F	FFPE tissue	NSCLC	GLY12CYS	10 %	10.6 %
5	1951	F	FFPE tissue	NSCLC	GLY12ASP	37 %	60.4 %
6	1958	F	FFPE tissue	NSCLC	GLY12VAL	4.1 %	3.3 %
7	1967	F	FFPE tissue	NSCLC	GLY12VAL	19.1 %	22.1 %
8	1983	M	FFPE tissue	NSCLC	GLY12ASP	37.2 %	42.4 %
9	1951	M	FFPE tissue	CRC	GLY12ALA	14.7 %	28 %
10	1959	M	FFPE tissue	NSCLC	GLY12CYS	29.5 %	66 %
11	1963	F	FFPE tissue	NSCLC	GLY12CYS	29.5 %	34 %
12	1956	F	FFPE tissue	CRC	GLY13ASP	33.6 %	44 %
13	1954	M	FFPE tissue	CRC	GLY12VAL	0.60 %	0.62 %
14	1945	M	FFPE tissue	NSCLC	/	0%	0.41 %
15	1944	F	FFPE tissue	CRC	/	23 %	25 %
16	1949	M	FFPE tissue	CRC	/	25 %	32.1 %
17	1958	M	FFPE tissue	CRC	/	20 %	23.2 %
18	1958	F	FFPE tissue	NSCLC	GLY12ALA	10.2 %	0 %
19	1952	F	FFPE tissue	CRC	GLY12VAL	36 %	38 %
20	1950	M	FFPE tissue	CRC	GLY12ASP	18 %	21.5 %

21	1952	M	FFPE tissue	NSCLC	GLY12VAL	24 %	28 %
22	1953	M	FFPE tissue	NSCLC	GLY12ASP	50.8 %	51 %
23	1952	F	FFPE tissue	NSCLC	GLY12ASP	21.6 %	13 %
24	1959	M	FFPE tissue	CRC	GLY12CYS	15 %	16 %
25	1953	M	FFPE tissue	CRC	GLN61HIS	8.1 %	/
26	1975	F	Plasma	NSCLC	GLY12CYS	4.1 %	3.2 %
27	1950	M	Plasma	NSCLC	GLY12ASP	9.4 %	10 %
28	1953	M	Plasma	NSCLC	/	ND	0.6 %

Mutational status

Out of a total of 28 samples, one mutation was detected in codon 13, Gly13Asp, six patients were characterized with Gly12Cys mutation, six patients exhibited mutation Gly12Asp, five of them had Gly12Val mutation and four of them had presence of Gly12Ala mutation. Based on the numerical values provided in the table, ddPCR exhibited higher sensitivity rate in majority of cases, which can also be observed in a Fig. 1 shown below.

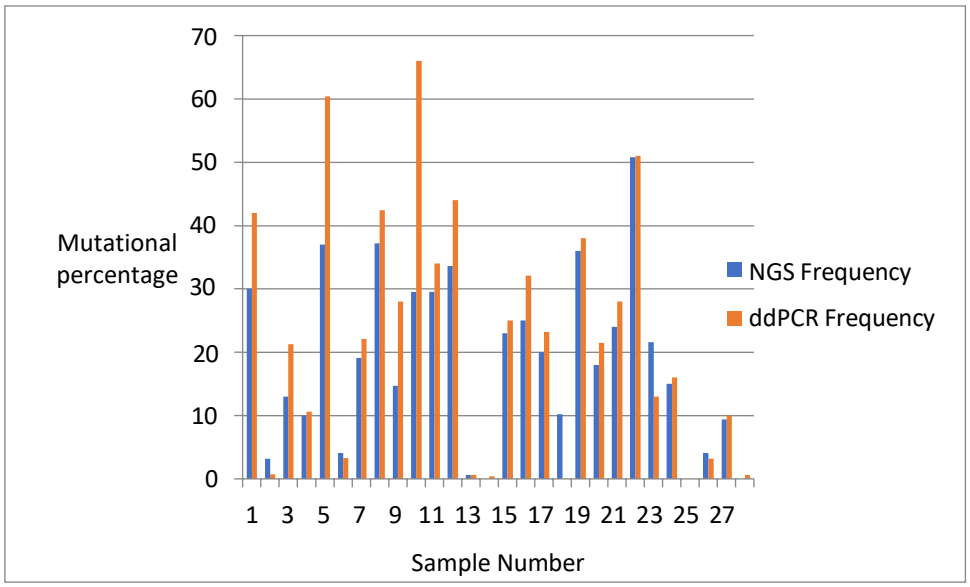

Figure 1. Mutation frequencies detected by ddPCR and NGS.

Statistical analysis

Using χ2 test for the observed mutations, we obtained the statistically significant results, with p – value being less than 0.05% . The chi square values obtained were 6.11 in case of CRC samples and 17.51 in case of NSCLC samples. Mean mutation frequencies were 20.74 and 23.61 for NGS and ddPCR results, respectively. Although ddPCR appears to be more sensitive when compared to NGS, the obtained p value was 0.53, which means that this difference in mean values was not statistically significant. The mean mutation frequencies between the two methods were also compared with respect to mutation type, cancer type, sample type as well as sex, but all the obtained p values were above the critical 0.05 level.

4. Discussion

In sample number 14, the patient tested negative for *KRAS* mutation by NGS and positive by ddPCR (0.41%). Taking into consideration that droplet digital PCR also detected low frequency mutation, NGS could not trace the mutation because the sensitivity level it usually detects is set at 1%. For sample number 18, NGS detected the mutation at 10.2% but ddPCR did not. Sample number 25 exhibited Q61 mutation, which could not be detected by ddPCR as expected since it is designed to trace exclusively *KRAS* G12/G13 mutations. Patient under sample numbered by 28 is the one who has already gone through the treatment process after being diagnosed with NSCLC, so the droplet digital PCR in this case was done with the goal of monitoring the eventual progress of the disease.

The comparison of different molecular methods used in detection of *KRAS* G12 / G13 mutational status has shown that ddPCR is more sensitive NGS. Besides droplet digital PCR and next generation sequencing represented in this work, the methods used in analysis process might also include Sanger sequencing, peptide nucleic acid – clamping (PNA clamping assay), and quantitative PCR.

In a study published by Kyung Ha Lee and colleagues, the identification of *KRAS* G12 / G13 mutational status in CRC patients was accomplished using ddPCR and compared to the results of Sanger sequencing and the PNA clamping assay.Compared to Sanger sequencing method and PNA-clamping assays, their study found that ddPCR retained high sensitivity and specificity (the percentage was 100% in both cases).When compared to NGS panel sequencing, ddPCR and Sanger sequencing also showed higher sensitivity expressing values of 96.43 % and 100 % respectively. When it comes to specificity, the obtained results were

98.11 % in case of ddPCR and 92.45 % in case of Sanger sequencing. NGS panel sequencing, on the other hand, allowed for scanning of numerous genes, including KRAS G12/G13 status, but demonstrated low sensitivity level and greater computation value [3]. Thus, ddPCR, Sanger sequencing and PNA-clamping assay showed comparable outcomes for *KRAS* G12 / G13 mutations. The quantity of DNA required for ddPCR was 1 L, which was significantly less than the amount required for Sanger sequencing (20 ng) and the PNA-clamping assay (7 l). This practicalbenefit is useful when detecting *KRAS* G12 / G13 mutations in both, biopsied tissues and liquid biopsy samples.Moreover, the *KRAS* G12 / G13 multiplex kit was unable to differentiate the mutation codon site and did not cover the entire spectrum of KRAS mutation sites. The ddPCR platform is equipped with two fluorescence filters and can perform at least duplex reactions [3].

Janku F. and others presentedcomparable results. They demonstrated that the *KRAS* G12 / G13 mutations from patients with advanced malignancies can be detected using ddPCR on a small amount of plasma cfDNA that has not been amplified, with satisfactory concordance of 85 %, sensitivity of 84 % and specificity of 88 % [15].

Advantages of NGS include detection of different types of mutations as compared to ddPCR which cannot, as shown in the example of Patient 25. On the other hand, ddPCR is good for detection of a specific mutation at a low frequency. In sample 18, we could not detect the mutation by ddPCR. Our results show that NGS and ddPCR are equivalent methods for the detection of the percentage of *KRAS* mutations.

5. Conclusion

Even though CRC and NSCLC remain as a primary reason of death among all cancer types, utilization of novel therapies based on EGFR inhibition, showed significant results and increased the survival rate among the patients.

In this study we investigated the sensitivity of two sophisticated diagnostic methods: NGS and ddPCR. Even though both methods can provide meaningful and robust diagnostic information, when comparing the methods, we found that ddPCR is more sensitive and superior then NGS, primarily due to its capability of tracing the *KRAS* mutation with higher specificity and accuracy.

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The Assessment of the General Health Status of the Sarajevo Canton Residents Through General Biochemical Testing Results

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Orginal research

Abstract: *In this research, we will be assessing the general health status of the Sarajevo Canton residents by analyzing the biochemical test results for the samples from the study cohort collected in 2021 for the following testing panels: lipid panel, liver panel, thyroid panel, electrolyte panel, and prostate panel. Along with the biochemical components that are tested, their physiological importance and consequences of deregulation, we are introducing the reasons for ordering the tests, as well as potential findings and conclusions from the testing results. Devices that were used for this research are Gesan Chem 200 (Gesan Production, Campobello di Mazara, Italy) and Architect 1000 (Abbot, Chicago, IL), both operated according to manufacturer's instructions. The collection of demographic data and test results was performed through usage of Laboratory Information System (LIS). Test results of the study participants were compared with referent values, as provided for the population average. Initial results were summarized and compared through descriptive statistics, while the exact test of goodness-of-fit was used to assess the compliance of observed data with expected referent values. Demographic trends, in terms of which sex and/or age group are more likely to have any tested parameter deregulated, either increased or decreased, are presented and discussed in detail. Finally, recommendations for future testing and prophylaxis are given.*

Keywords: laboratory testing, biochemical blood testing, Sarajevo Canton, health status

1. Introduction

Blood circulates through the human body and delivers essential substances like oxygen and other nutrients to the body cells. Main blood functions are supporting the immune system, transporting oxygen and regulating body temperature (Stroncek et al., 2013). Straw-colored clear liquid is called plasma, and it makes around 55% of the blood. Cellular elements and dissolved substances are suspended in plasma. After removing fibrin from blood, serum is the fluid portion of blood which remains. Plasma consists of 8% of combination of inorganic and organic substance and 92% of water. Blood consists out of three elements, red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (thrombocytes) (Stroncek et al., 2013).

Biochemical tests, which measure elements in blood and urine (such as protein, sugar, oxygen, etc.), are frequently used to diagnose illnesses and choose the best course of therapy (Joseph et. al., 2011). One or more of the specific biochemical indicators are impacted by the activity of each organ in the body. As a result, measuring concentrations and comparing biochemical indices in blood components can aid in the diagnosis of a wide range of illnesses (Joseph et. al., 2011).

Electrolytes are electrically charged minerals that aid in maintaining the proper balance of acids and bases in human body as well as fluid retention. They also support the regulation of heart rhythm, muscle and neuron activity, and other crucial processes (Gumz et al., 2015). A blood test called an electrolyte panel, often called a serum electrolyte test, analyzes the concentrations of the body's primary electrolytes: sodium, chloride, potassium, and bicarbonate. Any of these electrolyte imbalances may indicate a major health issue, such as kidney illness, hypertension, or a potentially fatal heart rhythm irregularity.

The term "renal" refers to the kidneys, which are the blood-filtering and -purifying organs. A panel test entails multiple measurements being taken from the same sample that offers detailed knowledge on the condition of the kidneys and can aid in the early identification, diagnosis, and monitoring of kidney issues (Boga et al., 2019). A blood sample is used by the thyroid panel to assess how well the thyroid gland is working, as well as to perform the diagnosis and management of thyroid diseases (Eggersten et al., 1988). The thyroid panel test, which is a collection of measurements, can give a thorough insight of how effectively the thyroid gland is functioning. It specifically calculates the body levels of thyroid hormones and thyroid-stimulating hormones, including TSH (thyroid-stimulating hormone), free T4 (thyroxine), and total T3 or T3 (triiodothyronine).

Blood tests called liver function tests, commonly referred to as liver panels, examine various enzymes, proteins, and other chemicals produced by the liver. These tests examine human liver overall condition, and may include the following components: albumin, total protein, bilirubin, lactate dehydrogenase, and prothrombin time.

A blood test called a lipid panel counts the number of certain fat molecules, or lipids, in human blood. The panel typically includes a test of human triglycerides, as well as four separate cholesterol measures, including total cholesterol, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) (Grundy et al., 2019).

The prostate panel (PSA) test is a blood examination used primarily for prostate cancer screening. The prostate-specific antigen (PSA) level in the blood is determined by the test. The prostate is a tiny gland that resides underneath the bladder in males (Dvorácek et al., 2019). Both malignant and noncancerous cells in the prostate produces the protein known as PSA. Semen, which is also produced in the prostate, is primarily where PSA is discovered. PSA often circulates in the blood in small levels. The PSA test may be utilized in men who have already received a prostate cancer diagnosis to analyze a treatment efficacy and monitor possible cancer recurrence.

2. Materials and methods

Prior to starting with data analysis, ethical clearance for conducting the study was obtained from the Ethics Committee of the Faculty of Engineering and Natural Sciences, International Burch University in Sarajevo, Bosnia and Herzegovina. The permission to conduct the research was granted on December 7th, 2021, document number 04-116-1/21. Samples are anonymized and no-one except for the primary investigators on the project was familiar with participant identity.

Blood samples have been collected from 102 patients, from January until December 2021, and a total of 135 panel analyses has been performed with these samples (Table 1). Devices that were used for this research are Gesan Chem 200 (Gesan Production, Campobello di Mazara, Italy) and Architect 1000 (Abbott, Chicago, IL), both of them taking a basic blood sample as an input material.

Table 1. The number of samples tested per study panel.

Panel	Number of samples
Prostate panel	15
Electrolyte panel	20
Thyroid panel	30
Liver panel	30
Lipid panel	40

Depending on the type of analysis being performed, different sampling procedure is employed. All panels share certain similarity in process, whereby the amount of blood needed is 3.5 mL or 5 mL, depending on the amount of serum required. Collected sample should rest anywhere between 15 and 20 minutes for blood to coagulate. The sample is then centrifuged for 10 minutes on 3,000 rpm to get the serum. After this, we approach the analytical analysis of specimen. For the lipid, electrolyte, and liver panels, Gesan Chem 200 device and suitable reagents were used, according to manufacturer's instructions. Prostate and thyroid panel are performed on Architect 1000 device and using adequate reagents, according to manufacturer's instructions.

The collection of demographic data and test results were performed through usage of Laboratory Information System or LIS. Test results of the study participants were compared with referent values, as provided for the population average. Initial results were summarized and compared through descriptive statistics, while the exact test of goodness-of-fit was used to assess the compliance of observed data with expected referent values.

3. Results and Discussion

Thyroid Panel

For the analysis of this panel, 30 samples were collected, all of them from females. In order to examine the effect of age on the test results, study participants were divided into four groups (Figure 1) and data analyzed separately. The age of study participants ranges from 19 to 72, with the average age of 42.93. Samples were divided among four groups as follows: group 1, aged 19-30, n=6 (20%); group 2, aged 31-40, n=10 (33.33%); group 3, aged 41-50, n=6 (20%); and group 4, participants older than 50, n=8 (26.67%).

Regarding TSH analysis, participants in age groups 1 and 4 were not found to

have TSH levels outside of the reference range. In group 2, one participant was above and one was below the reference range. The same situation was observed in group 3. The exact test of goodness-of-fit has shown that there is no significant difference between age groups 2 and 3 (20% and 33.33%, respectively; p=0.098). Age groups 2 and 3 were also found to be significantly more likely outside of the reference range when compared to groups 1 and 4, with a p value of 1.91×10^{-6} for age group 2 when compared to groups 1 and 4 and a p value of 2.33×10^{-10} for age group 3 when compared to groups 1 and 4.

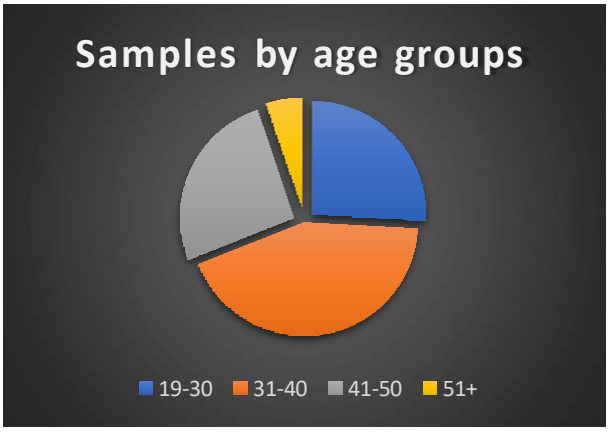

Figure 1. Samples analyzed for the thyroid panel divided into four age groups.

Regarding T3 and T4 analysis, the results were the same for these two parameters. There were no participants with results outside the reference range in groups 1, 2 and 4. In group 3, one participant had increased values for both T3 and T4 (16.67% of all participants in group 3). Therefore, group 3 participants were significantly more likely to have deregulated T3 and T4 levels when compared to any other study group (p=0.000031).

Prostate Panel

Opposite to the thyroid panel, all samples collected here came from male population, with a total of 15 samples being analyzed in the present study for total PSA and free PSA measurements. Their age was ranging from 31 to 89, with an average of 58.87 and standard deviation of 16.17 years. We approached this analysis by dividing samples by age groups, whereby group 1 consists of individuals aged between 31 and 40 (n=3, 20% of all participants), group 2 of individuals aged between 41 and 60 (n=4, 26.67%), group 3 contains individuals aged from 61 to 70 (n=4, 26.67%), while group 4 has individuals older than 70 (n=4, 26.67%) (Figure 2).

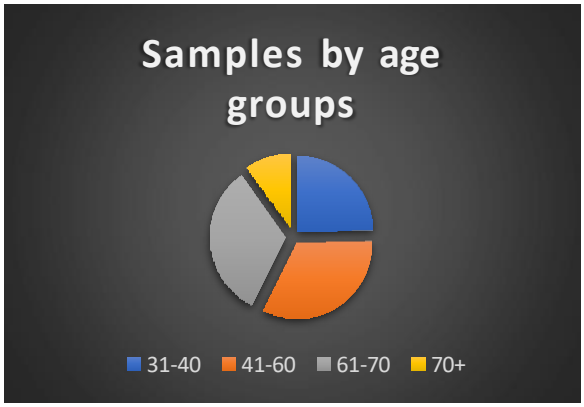

Figure 2. Samples analyzed in the prostate panel separated by age groups.

Regarding free PSA analysis, all participants had their results within optimum range. As for the total PSA, five participants had increased values, including one participant in each of groups 1, 3 and 4, as well as two participants in group 2. Therefore, in group 1, 33.33% of participants had increased total PSA, which is not statistically significant when compared to group 2 (p=0.078), or groups 3 and 4 (p=0.358). In group 2, 50% of participants had increased total PSA, which was not significantly more when compared to group 1 (p=0.078) but is when compared to both groups 3 and 4 (p=0.005228). Groups 3 and 4 had 25% participants each with increased total PSA (p=1 for group 3 vs. group 4) (Figure 3).

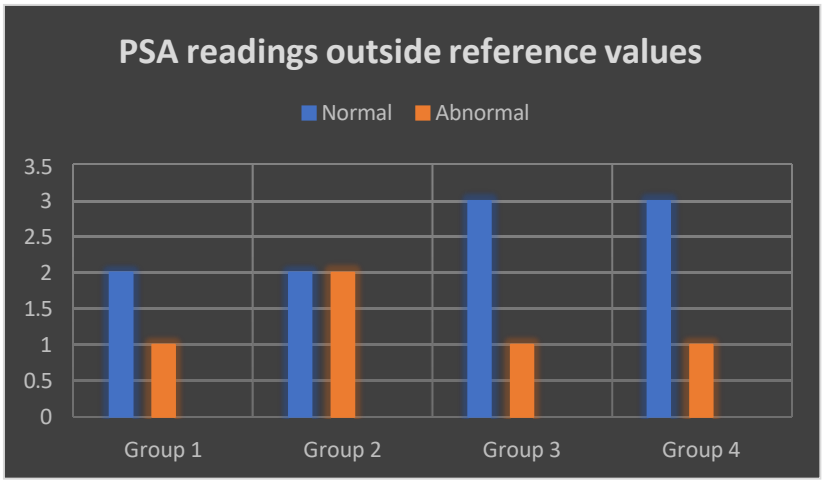

Figure 3. Total PSA readings within and outside the referent values, separated into four age groups.

Liver Panel

In liver panel, we performed 30 tests, with 15 male and 15 female participants. Mean age of all participants was 42.1, ranging from 24 to 82. Standard deviation was 14.47

years. When observing females only, average age was 44.1, while for males, average age was 40.13.

In female group, only one participant (6.67% of all females) had GGT increased, while the results for AST, ALT, ALP and LDH were all within the recommended range. For males, two participants (13.33% of all male participants) had increased AST, four (26.67%) had increased ALT, while three had increased each of ALP, GGT and LDH (20% for each parameter). Therefore, males were statistically more likely to have any of these parameters increased than females, with p values of 0.000224 for AST, 2.98×10^{-8} for ALT, 1.91×10^{-6} for ALP, 0.009355 for GGT, and 1.91×10^{-6} for LDH.

Next, we have divided all our participants in four age groups, regardless of sex, as follows: group 1 aged 30 and younger (n=6, 20%), group 2 aged 31-35 (n=9, 30%), group 3 aged 36-50 (n=7, 23.3%), and group 4 aged 51 and older (n=8, 26.67%) (Figure 4).

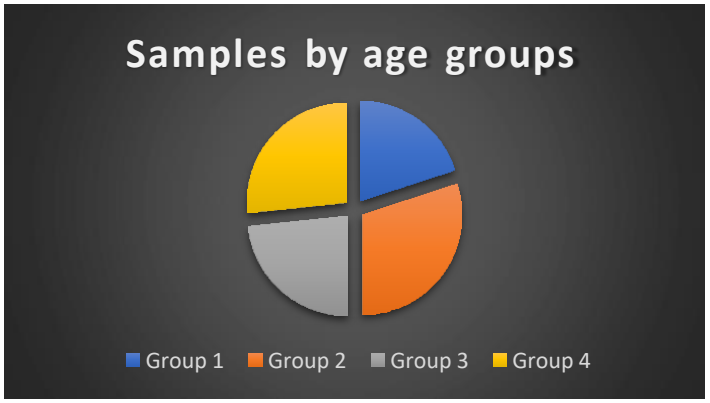

Figure 4. Liver panel samples by age groups.

For AST analysis, there was one increased result in each of groups 1 and 2 (16.67% for group 1 and 11.11% for group 2), which was not statistically significant (p=0.442). There were no increased results in groups 3 and 4. Therefore, group 1 was more likely to have increased AST when compared to groups 3 and 4 (p=0.000031), as well as group 2 when compared to groups 3 and 4 (p=0.000997). In ALT analysis, there were two increased values in each of groups 1 and 2 (33.33% for group 1 and 22.22% for group 2) and no results outside the reference range in groups 3 and 4. Groups 1 and 2 were not significantly different from each other (p=0.177). Group 1 participants were more likely to have increased ALT when compared to groups 3 and 4 ($p=2.33 \times 10^{-10}$), just like group 2 participants when compared to groups 3 and 4 ($p=4.77 \times 10^{-7}$). ALP and LDH

analysis offered the same results in that one participant (16.67%) from group 1 had both parameters increased, as well as two participants (22.22%) in group 2. There were no such measurements in groups 3 and 4. There was no significant difference in the frequency of increased ALP and LDH between groups 1 and 2 ($p=0.418$). There was, however, significantly more increased ALP and LDH in group 1 when compared to both groups 3 and 4 ($p=0.000031$), as well as in group 2 compared to groups 3 and 4 ($p=4.77 \times 10^{-7}$). Finally, when analyzing GGT, we observed one participant with increased value in groups 1 and 4 (16.67% for group 1 and 12.5% for group 4), two (22.22%) in group 2 and 0 (0%) in group 3. Group 1 did not significantly differ from group 2 ($p=0.418$) or group 4 ($p=0.458$) but did differ from group 3 ($p=0.000031$). Group 2 also did not differ from group 4 ($p=0.121$) but did differ from group 3 ($p=4.77 \times 10^{-7}$). Groups 3 and 4 did differ statistically ($p=0.000448$) in that group 4 participants were more likely to have increased GGT when compared to group 3.

Electrolyte Panel

Regarding electrolyte panel, total number of samples was 20, equally divided among males and females. Average age of panel participants was 49.95, ranging from 20 to 83 and with standard deviation of 19.06.

Regarding abnormal values in these readings, we only noticed that 10% of females (n=1, 73 years old) had decreased level of calcium, while all other readings were regular. When it comes to male population, we noticed that 10% had decreased sodium (n=1, 58 years old), chlorides (n=1, 63 years old), and calcium (n=1, 70 years old). Finally, one 57-year-old male (10% of all males) had slightly increased potassium levels.

Statistical overview of data outside of the expected range by sex and corresponding measurements, including p values is presented in Table 2 below. There is no significant difference between males and females when it comes to Ca ($p=1$), while males are more likely to have other parameters (Na, K, and chlorides) deregulated when compared to females ($p=0.001953$ in all three cases).

Table 2. Electrolyte panel samples with values outside the referent range and statistical significance between the sex groups.

Na	K	Chlorides	Ca	
0%	0%	0%	10%	Percent female
10%	10%	10%	10%	Percent male
0.001953	0.001953	0.001953	1	p value

This analysis also differs between two age groups, namely G1 with 9 samples with age below 50, and G2 with 11 samples and age above 51. Regarding the differences in electrolyte level deregulation between the age groups (Table 3), we can see the G2 is significantly more likely to have any of these individual measurements deregulated when compared to G1 ($p=0.003906$ for Na, K, and chlorides, and $p=7.63 \times 10^{-6}$ for Ca).

Table 3. Statistics comparison between measurements and age groups including p values.

Na	K	Chlorides	Ca	
0%	0%	0%	0%	G1
9.09%	9.09%	9.09%	18.18%	G2
0.003906	0.003906	0.003906	7.63×10^{-6}	p value

Lipid Panel

Lipid panel contained 40 samples, divided in two groups of 20 males and 20 females. Age was ranging from 24 to 85, with an average value of 51.1 years of life. We additionally divided samples into four age groups (Figure 5), whereby group 1 (G1) contains samples from individuals aged 40 or younger (n=10, 25% of all study participants), group 2 (G2) individuals from 41 to 45 years (n=12, 30%), group 3 (G3)

individuals from 46 to 65 (n=10, 25%), and group 4 (G2) individuals aged 66 or older (n=8, 20%).

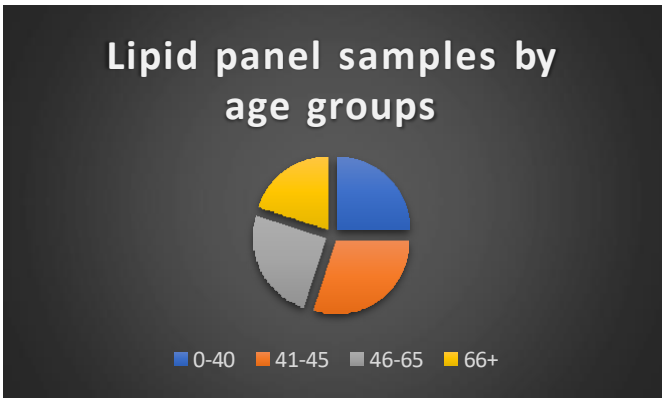

Figure 5. Lipid samples by age groups

For cholesterol measurement, G3 had most values out of expected range (90%), G1 showed 50% out of expected range, while G2 and G4 showed 41.67% and 25% values outside referent values, respectively. What we can notice for HDL statistics is that none of the values were higher than expected, only G1 and G2 in 10% and 16.67% cases, respectively, exhibited samples with value lover than referent ones. Opposite to the HDL readings, none of the samples in LDL data were lower than referent values, G4 and G3 in 50% of their samples showed results higher than expected, while G2 and G1 exhibited such samples in 41.67% and 20% of all cases, respectively. Similar to LDL data, none of the tested samples displayed triglyceride levels below the referent range. As for the readings above the referent range, G3 had the highest frequency of those readings (50% of all samples), followed by G4 (37.5%) and G1 (30%), while the lowest frequency was in G2 (25%). These results are shown in Table 4 below.

Table 4. Overview of lipid panel data separated by age groups.

Out of range	Higher	Lower	Group
Cholesterol			
50%	20%	30%	G1
41.67%	25%	16.67%	G2
90%	50%	40%	G3
25%	0%	25%	G4
HDL			
10%	0%	10%	G1
16.67%	0%	16.67%	G2
0%	0%	0%	G3
0%	0%	0%	G4

LDL			
20%	20%	0%	G1
41.67%	41.67%	0%	G2
50%	50%	0%	G3
50%	50%	0%	G4
Triglycerides			
30%	30%	0%	G1
25%	25%	0%	G2
50%	50%	0%	G3
37.5%	37.5%	0%	G4

Next, we separated our study population by sex, into male and female groups. Female samples had abnormal value readings in the following categories: cholesterol 55%, LDL 40% and triglycerides 35%. Regarding male samples, we calculated the following values: cholesterol 50%, HDL 15%, LDL 40%, and triglycerides 35%. If we reflect on p values, we come up with following results: p=0.696 for cholesterol, p=0.000061 for HDL, p=1 for LDL, and p=1 for triglycerides. This means that HDL was significantly more likely to be deregulated, either increased or decreased, in males when compared to females, while those differences for other parameters were not significant. Figure 6 displays comparison of normal readings and readings outside expected value for male samples, while Figure 7 shows the same data for female samples only.

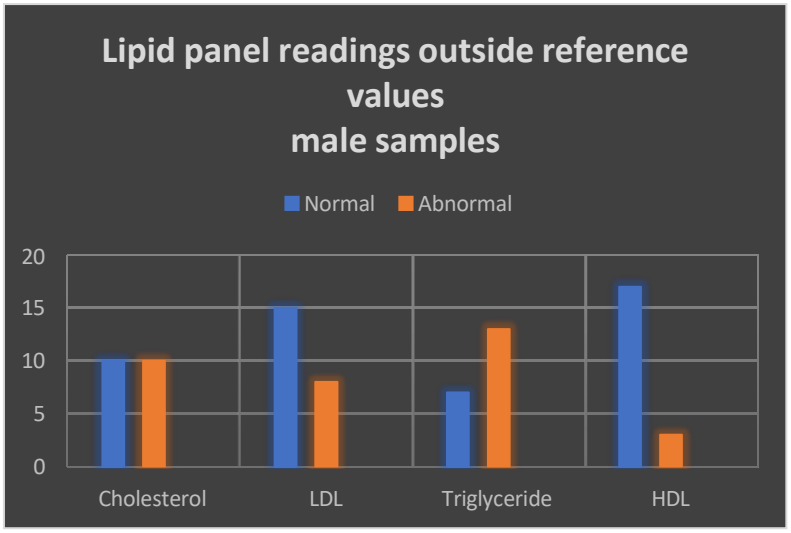

Figure 6. Lipid male samples with readings within and outside referent interval.

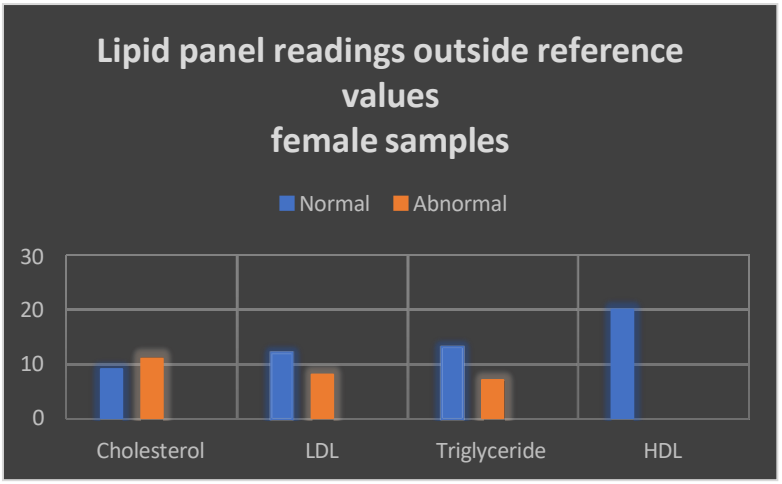

Figure 7. Lipid panel readings within and outside referent values for females.

If we only consider the case in which sample values were above the referent range, for male samples we get the following frequencies: cholesterol 25%, LDL 40%, triglycerides 35%; and for female samples cholesterol 25%, LDL 40%, and triglyceride 35%. Since p values in all cases are equal to 1, it means that there are no differences between male and female groups.

Opposite to previous observation, we next considered only those cases where readings were below the referent values. For female samples, we got that only cholesterol was below the referent values in 30% of cases. Regarding male samples, readings for cholesterol and HDL were in 25% and 15% cases below referent values, respectively. p values in these cases are: p=0.59 for cholesterol, p=0.000061 for HDL, and p=1 for both LDL and triglycerides. This means that males are significantly more likely to have HDL values below referent interval than females, while other differences did not reach statistical significance.

4. Discussion

To be able to assess the general health of a certain group or a general population, the number of samples, period in which samples were collected, and reason for testing, and demographic characteristics of a study cohort all play a major role in objectivity of results and determine how representative the study sample is of the general population. We should not neglect the fact that samples which were analyzed in the present study came from both patients who were referred to examination by their physician or self- initiated the testing. Improvements to the present study and better application of obtained results could be obtained by considering only those samples that came from patients that were analyzed based on self-initiative, because usually the patients which are referred by the medical doctors, are not the ones which represent general health in the best possible

manner. In addition, it would be beneficial to increase the number of samples per panel, achieve well-balanced sex and age group representation, as well as analyze additional blood parameters, in order to get more meaningful results. We should also keep in mind that samples were collected during the second year of COVID-19 pandemic, which potentially also influenced some of the obtained readings.

5. Conclusions

The general conclusion of the present research is that the citizens of the Canton Sarajevo are the most likely to be affected by problems regarding lipid components of blood, more precisely increased LDL, cholesterol and triglycerides in blood. We can conclude that Canton Sarajevo population should be monitoring their lipid levels more closely in order to avoid associated problems, such as cardiovascular disease and metabolic syndrome. Statistical data analysis also gave us information that males are more likely to have problem in any of the tested categories, except for thyroid gland hormone levels, which was tested in female participants only. Considering that only female samples were available for the thyroid panel, implies that males are not likely to get tested for those components, which is a practice that should be changed in close future.

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